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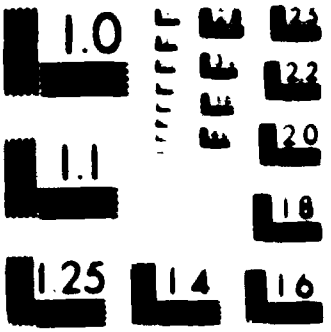
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This research program explores the influences of neuroendocrine mediators on macrophage functions. Specifically we have found that met-enkephalin and substance P enhance antibody dependent phagocytosis of sheep red blood cells while epinephrine depresses phagocytosis. Many experimental results have been observed for both the RAW264 macrophage cell line and peritoneal exudate macrophages. In the complex physiological environment phagocytic cells receive many simultaneous but distinct ligand-receptor signals. To more closely reflect this environment *in vitro* we have examined the effects of simultaneous treatment with met enkephalin and epinephrine on RAW264 macrophage phagocytosis and cytolysis. We have discovered that at their physiologically-relevant doses these two ligands profoundly depress phagocytosis. At the several doses tested the combined effects of these two ligands on the amount of phagocytosis were equivalent to or less than that of epinephrine alone. Therefore *in vitro* models more closely approximating *in vivo* neuroregulation of macrophage function demonstrate phagocytic inhibition.

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ANNUAL REPORT

Influence of Neuroendocrine Mediators on Phagocyte Function
ONR Contract No. N00014-86-K-0634

1. Introduction

Psychosocial and environmental stressors can affect the immunological response. Mice subjected to standardized avoidance-learning type of stress have a prolonged survival of skin homografts (1). Enhancement of the immune response has also been observed. For example, brief handling of newborn rats increased both primary and secondary antibody responses to challenge with flagellin (2). The neuroimmunologic mechanisms responsible for these observations are not known. Phenomenological studies employing experimentally-induced lesions of the nervous system have provided some insight into these mechanisms. Hypothalamic lesions have led to depressed delayed cutaceous reactions to picryl chloride and tuberculin accompanied by decreased titers of anti-picryl hapten antibodies (3). Similarly, such lesions in rabbits lead to decreases in the levels of complement-fixing anti-horse antibodies (4). Recent studies have illuminated several aspects of stress-induced modulation of lymphocyte function (5-10). Inescapable, but not escapable, shock suppresses lymphocyte proliferation (5). Furthermore, immediate hypersensitivity responses, as measured by plasma histamine levels, can be learned in classical conditioning methodologies (11). A neuro-endocrine-immune system regulatory axis has been proposed (12). The interactive chemical signaling between components of the super-system is complicated by the cellular heterogeneity and dissemination of immune effector cells throughout the body. In vitro studies of immune cell function have been illuminating. The stress-related hormones adrenocorticotropin (ACTH), noradrenaline (norepinephrine), adrenaline (epinephrine), and glucocorticoids suppress several lymphocyte responses including proliferation, lymphokine and antibody production (13,14). Endogenous opioids modulate mitogenic stimulation of T cells (16) and influence cytotoxicity of NK cells (17). Furthermore, T cells possess a cell surface component similar or identical to the nicotinic acetylcholine receptor (18). The principle focus of research on the neuro-endocrine-immune system has been lymphocytes. However, macrophages and polymorphonuclear (PMN) leukocytes form the first line of host defense against infectious agents. Phagocyte function can be affected by the neuroendocrine mediators previously studied in the lymphatic arm of the immune system (19-31). Vasoactive intestinal polypeptide and somatostatin can be found within PMNs and mononuclear leukocytes (34-36). β-endorphin has been found in association with a subpopulation of spleen macrophages (38). Substance P and neurotensin have been found to influence phagocyte function (39,40). An understanding of the roles of neuroendocrine mediators on host resistance to infection requires and analysis of phagocytic cells. The goal of this project is to illuminate several key interactions of neuroendocrine mediators with host phagocytic effector cells.

2. **RESEARCH OBJECTIVE (YEAR 1):** The principal objective during year 1 was to investigate the effects of neuroendocrine mediators on in vitro macrophage effector functions.

3. **PROGRESS REPORT**

This progress report covers the year from July 1, 1986 to June 30, 1987. In this period of time we have obtained very solid and interesting results regarding in vitro influences of neuroendocrine mediators upon phagocyte effector functions. Since our future experiments depend critically upon these

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studies, we have adopted a fairly conservative approach in these studies. For example, instead of performing many kinds of assays in a superficial fashion, we have made in depth studies of just phagocytosis and cytolysis. Moreover, we have discovered novel combinative effects of simultaneous ligation of opioid and adrenergic receptors.

A. Measurement of Phagocytosis and Cytolysis

During the first two to three week period of this contract we set-up and tested the ^{51}Cr assays for antibody-dependent phagocytosis and cytolysis. Previously established methods have been employed. For examples see Figures 1 and 2. The phagocytic targets employed in this assay are ^{51}Cr labeled sheep erythrocytes. Erythrocytes opsonized with antibody are referred to as EAs. Labeling with ^{51}Cr had no detectable effect upon erythrocyte morphology or viability as measured by retention of hemoglobin and exclusion of trypan blue. Incubation of EAs with macrophages did not influence macrophage viability. EAs can be bound, lysed, or phagocytosed by macrophages. These distinct effects can be quantitatively measured by the ^{51}Cr assay. Lysed cells release label in to the supernate. Bound, but not phagocytosed EAs, are eliminated by brief exposure to distilled H_2O. Phagocytosed label can be released by detergent treatment. As a control, antibody can be omitted; this controls for non-specific uptake. Phagocytosis is temperature-sensitive, saturable, and time-dependent. These three features would be expected if phagocytic activity were occurring. This has been confirmed with the ^{51}Cr-assay. At 4°C phagocytosis is inhibited. Uptake of EAs is time-dependent; it saturates at roughly four hours.

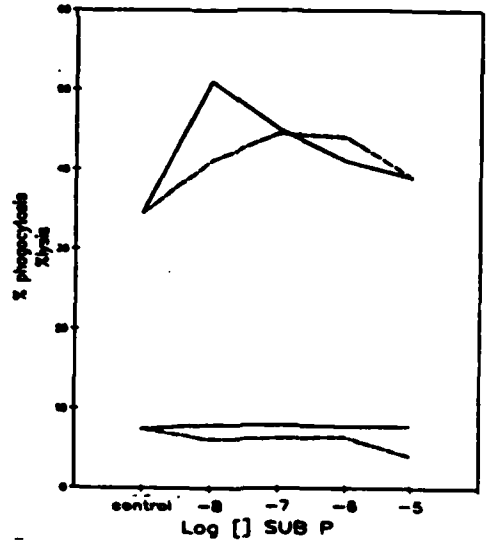

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Figure 1. The effect of substance P upon antibody-dependent phagocytosis and cytolysis of EAs by elicited murine peritoneal macrophages is shown. The data points represent the mean of duplicate determinations. Phagocytosis (_____) and cytolysis (.....) were measured after a one hour pulse with substance P. In addition the phagocytosis (--------) and cytolysis (-·-·-·-·-) assays were conducted in the presence of ligand. The ordinate is the percentage of phagocytosis or cytolysis while the abscissa is the dose of substance P. One of six similar experiments is shown. (Total phagocytosis, specific + non-specific, is shown. Non-specific phagocytosis is typically 5-8%)

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B. Substance P

One site of communication between the nervous and immune systems is found in the interaction of the peripheral nervous system (PNS) with local immunocompetent cells. Substance P is an undecapeptide that may act as a messenger between the PNS and local immunologic responses. The ability of substance P to augment the respiratory burst led us to propose that antibody-dependent phagocytosis may be similarly enhanced. This hypothesis has been tested. In Figure 1 we show the results of a representative substance P experiment. The ordinate gives the percentage of antibody dependent phagocytosis or cytolysis while the abscissa gives the dose of substance P. One of six similar experiments is shown. Two methods of macrophage incubation with substance P were performed. Macrophages were either: (a) pretreated with peptide for 1 hour at 37°C or (b) incubated with peptide for 1 hour with addition of fresh peptide during assay with ^{51}Cr-labeled RBCs. Although no significant effect upon cytolysis could be found, both incubation methods demonstrated enhanced antibody-dependent phagocytosis at physiological concentrations of substance P. Both RAW264 and elicited peritoneal macrophages demonstrate this response. Substance P concentrations of 10^{-10} and 10^{-9} M had no effect upon phagocytosis or cytolysis. Maximal stimulation was in the range 10^{-8} to 10^{-7} M peptide. The maximal increase at 10^{-8} M was 46%. Furthermore, this augmentation of phagocytosis is diminished if catalase is added to the medium. This suggests that stimulation of the respiratory burst and augmentation of phagocytosis may be related.

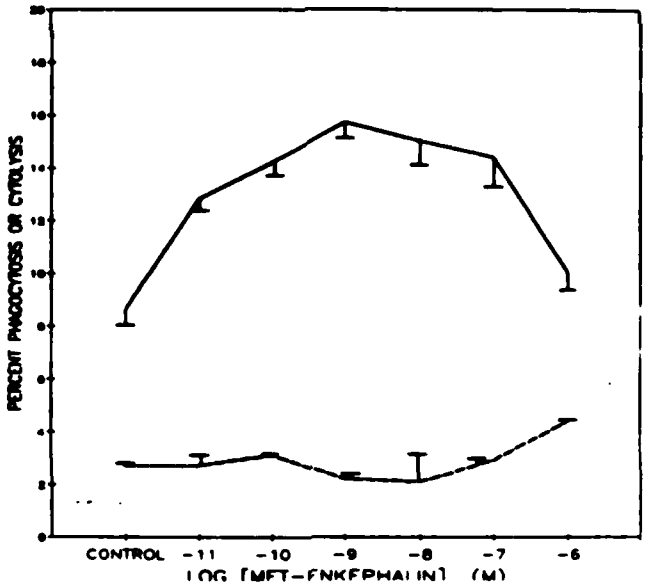

Figure 2. Representative dose-response curves illustrating the effects of met-enkephalin on specific antibody-dependent effector functions of RAW264 macrophages. The percent phagocytosis (_____) and cytolysis (---------) are shown at the ordinate. The molar concentration of met-enkephalin is given at the abscissa. One of six similar experiments is shown (n=3; ± s.e.).

C. Met-Enkephalin

In Figure 2 is shown a representative dose-response study of the

effect of met-enkephalin on antibody-dependent phagocytosis and lysis of sheep red blood cells. The abscissa is the molar concentrations of met-enkephalin while the ordinate is the percentage of phagocytosis or cytolysis of target cells. This is one of seven similar experiments; all studies showed enhanced phagocytosis with no significant effect upon cytolysis. This dose-response curve shows specific antibody-dependent effector functions. Non-specific phagocytosis and cytolysis were subtracted from data by performing similar experiments in the absence of antibody. In general we have found the contribution of non-specific phagocytosis to the 5-7% and non-specific cytolysis to be 2-4%. We have found met-enkephalin to have an optimal phagocytosis-enhancing dose of 10^{-9} to 10^{-8} M. Both higher 10^{-6} M and lower 10^{-12}M doses have little or no effect upon phagocytosis. Cytolysis was not influenced by met-enkephalin. Similar results were found using elicited murine peritoneal macrophages.

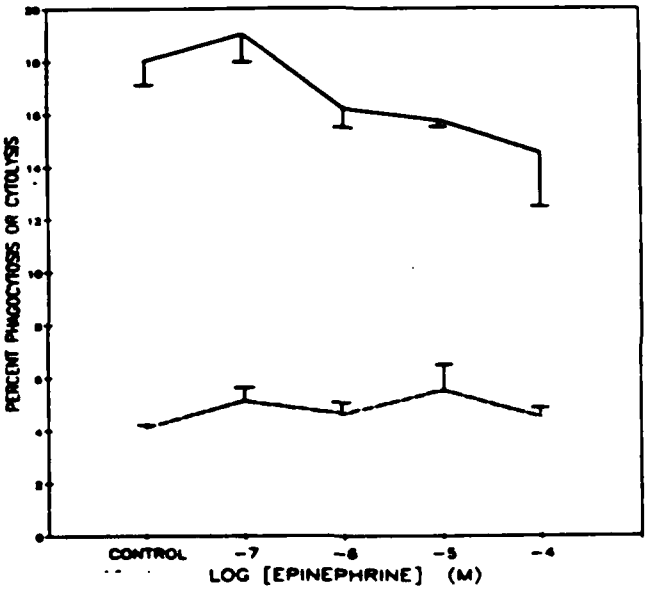

Figure 3. Representative dose-response curves demonstrating the effects of epinephrine on antibody-dependent effector functions of RAW264 macrophages. The percent specific phagocytosis (_____) and cytolysis (---------) are shown on the ordinate. The molar concentration of epinephrine is given at the abscissa. One of three similar experiments is shown (n=3; mean ± s.e.).

D. Epinephrine

Figure 3 shows the effect of epinephrine on antibody-dependent effector mechanisms of RAW264 macrophages. As described for Figure 1, the abscissa and ordinate are the molar concentration of epinephrine and the

percent phagocytosis or cytolysis, respectively. One of three similar experiments is shown. A dose of 10^{-5} to 10^{-4} epinephrine was found to maximally depress phagocytosis. Again, no significant changes in cytolysis were noted. Although the effects of epinephrine on target cell phagocytosis have not previously been measured, the optimal inhibitory dose does correspond to the optimal cAMP stimulation dose. The inhibitory effect was blocked by the simultaneous addition of the antagonist propranolol at 10^{-5} M.

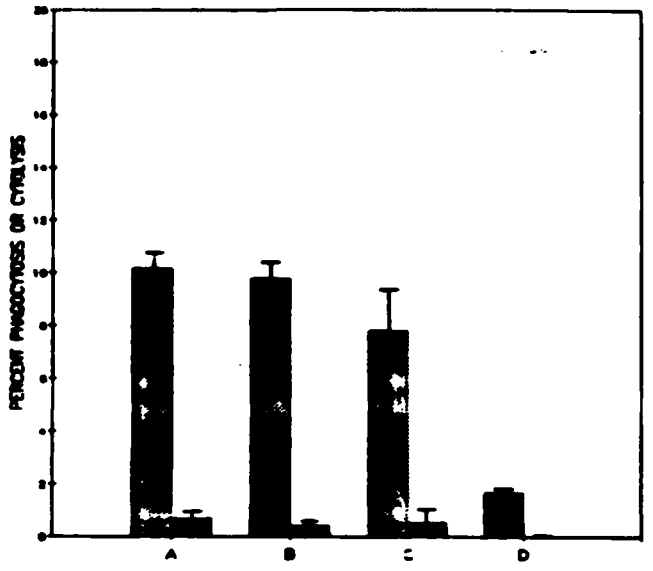

Figure 4. Representative data from combinative experiments using RAW264 macrophages are shown. The percent specific phagocytosis (cross-hatched bars) and cytolysis (solid bars) are given at the ordinate. The four sets of conditions are designated A through D. These conditions are the addition(s) of: A, buffer only or "control"; B, 10^{-8} M met-enkephalin and 10^{-8} M epinephrine; C, 10^{-6} M met-enkephalin and 10^{-6} epinephrine; and D, 10^{-8} M met-enkephalin and 10^{-5} M epinephrine. No lysis could be detected in samples D. One of five combinative studies are shown (n=3; mean $\pm$ s.e.).

E. Combinative Studies

Chromaffin cells of the adrenal synthesize met-enkephalin and leu-enkephalin (41). These opioid peptides are stored and secreted together with epinephrine from the adrenal medulla. To more closely approximate their physiological properties, we have measured the combined effects of both met-enkephalin and epinephrine on macrophage effector function. These studies indicated that the simultaneous presence of both ligands inhibits phagocytosis.

At all doses tested met-enkephalin stimulated or had no effect on phagocytosis. Similarly, epinephrine depressed or had no effect on phagocytosis. We have examined the effect of simultaneous treatment of RAW264 macrophages with met-enkephalin and epinephrine on phagocytosis and lysis. Figure 4 shows representative data from one of five combinative studies. This figure summarizes the central observations of these experiments. In each of these

trials three types of control experiments were performed. Control studies utilizing only added buffer are designated as "control". Furthermore, positive and negative controls were performed during every trial by adding only met-enkaphalin or epinephrine to separate assays. In all cases experiments were performed in triplicate. The several positive and negative controls applicable to the data of Figure 4 are not shown because they duplicate the results of Figures 2 and 3. The in vitro conditions we have employed had little or no effect upon lysis. However, specific antibody-dependent phagocytosis was significantly affected. Met-enkephalin at optimal doses has little ability to enhance phagocytosis in the presence of sub-optimal doses of epinephrine. The presence of met-enkephalin and epinephrine at their optimal doses profoundly diminishes phagocytosis ($P = 0.001$). All combinative experiments exhibited reduced specific phagocytosis in comparison to either controls or samples treated with only a matched dose of met-enkephalin.

F. Publications

H.R. Petty and K.A. Berg (1987). Combinative Ligand-Receptor Interactions: Epinephrine Depresses RAW264 Macrophage Antibody-Dependent Phagocytosis in the Absence and Presence of Met-Enkephalin. J. Cell. Physiol. submitted.

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